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Natural Antioxidants Dietary and Lipid Oxidation Analysis in Zebrafish (*Brachydanio rerio*) Tissue

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Autoxidation of lipids is related to flesh deterioration and discoloration, as well as unpleasant flavors produced during processing and storage. This becomes a major factor determining fresh and frozen seafood products price. The aims of this research were to investigate the effect of two novel dietary supplementation antioxidants Vinlife® grape seed extract and Herbalox® rosemary extract on lipid oxidation during post-mortem storage of zebrafish (*Brachydanio rerio*) and the relationships between dietary antioxidants with the enzymatic antioxidant activities. Herbalox® supplemented diet showed no harmful effects on growth or survival of zebrafish. However, the diet could not inhibit Thiobarbituric Acid Reactive Substances (TBARS) formation in zebrafish during post-mortem storage at 4 ºC. Dietary Herbalox® and Vinlife® supplementation also showed no deleterious effects on growth or survival of zebrafish. The Vinlife® supplementation efficiently suppressed TBARS formation in the zebrafish but the Herbalox® supplementation. The two novel antioxidants had significant effects on catalase and glutathione S-transferase but not on glutathione peroxidase. These findings suggested that the supplementation with Vinlife® was more effective than with Herbalox® in inhibiting the formation of lipid oxidation products in zebrafish. Hence, Vinlife® supplementation, could be a safe alternative method to improve oxidative stability of fish lipid contents.

Key words: natural antioxidant, lipid oxidation, TBARS, GPx, CAT, GST

INTRODUCTION

In aerobic organisms, oxygen is an essential element for cells to maintain normal body function and metabolism. However, oxygen also can give rise to reactive oxygen species (ROS) such as the superoxide radical (O_2^-), the hydroxyl radical (OH^-), the hydroperoxyl radical (OOH^-) and hydrogen peroxide (H_2O_2). In the body, the main source of ROS is cellular respiration, which involves mitochondrial electron transport (Bell *et al.* 1985; Mates *et al*. 1999; Rani *et al*. 2004; Norihiro 2005). Oxidative stress can occur when the generation of ROS exceeds the ability of antioxidant defense systems to neutralize or eliminate ROS (Sies 1985; Agarwal *et al*. 2003). Excessive production of ROS results in damage to various biological molecules such as nucleic acids, lipids, protein, and carbohydrates. Reactive oxygen species can attack and damage cell membranes and the lipoproteins through a process called lipid peroxidation (Martinez-Cayuela 1995).

Under aerobic conditions, all cells possess antioxidant defense mechanisms, which are divided into two groups: enzymatic and non-enzymatic antioxidants (Sharma & Agarwal 1996; Rani *et al.* 2004). A variety of enzymes is involved in antioxidant protection inside cells. These endogenous antioxidants act through dismutation, decomposition, and detoxification processes. They are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST). The main enzymes which help to detoxify ROS in all organisms are GPx and CAT. All of these enzymes are found in fish tissues (Di Giullio *et al*. 1993). The level of enzyme activity varies with species and muscle type (Decker & Xu 1998). In addition to the antioxidant enzymes, there are low molecular weight antioxidants, which act as scavengers. These include glutathione (GSH) and lipid- and water-soluble antioxidants. The lipid-soluble antioxidants include vitamin E and beta-carotene (vitamin A precursor), whereas vitamin C is one water-soluble antioxidants (Halliwell & Gutteridge 1989; Van Acker *et al*. 1993; Sharma & Agarwal 1996; Hayes & Mc Lellan 1999).

In general, fish fats contain higher levels of n-3 fatty acids compare to mammals but these very long-chain n-3 PUFAs contain many double bonds between carbon atoms in the fatty acid structure. This makes them highly susceptible to autoxidation. More number of double bonds fatty acids contain in the fatty acid, makes them more susceptible to autoxidation. In the absence of suitable antioxidant protection, high concentrations of PUFAs in fish flesh increase the amount of substrate for autoxidation. Hence, the risk of the fatty acids becoming rancid during processing and storage (Bell *et al*. 1985; Jensen *et al.* 1998). Autoxidation of lipids is related to flesh deterioration and discoloration, and the production of unpleasant flavors during processing and storage. This becomes a major factor determining the sales price of fresh and frozen seafood products. Therefore, efforts to reduce the rate of formation of rancid flesh are of great

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importance to the fresh and frozen fish and other seafood industries. This study was conducted to investigate the effect of novel dietary antioxidants in delaying lipid oxidation in zebrafish tissues.

MATERIALS AND METHODS

Experimental Fish and Diet Trial Condition. The protective effects of the novel antioxidants were investigated in two separate trials. In each trial, a standard commercial micropellet diet (proximate compositions were shown in Table 1) was used as the basal diet for the formulation of the three experimental diets. In Trial 1, a Herbalox® supplemented diet (oil-soluble form) was tested and compared with the standard diet. In Trial 2, two types of novel antioxidants were used Herbalox® (water-dispersible form) and Vinlife®. The effectiveness of these two antioxidants was investigated and compared with the standard diet. Each trial consisted of four replicate tanks for the standard diet and four replicate tanks for each antioxidant supplemented diet. Each tank for Trial 1 contained five fish, while in trial 2, each tank contained seven fish. These two trials were performed sequentially one after the other.

At the start of Trial 1, eight groups of five fish each, with an average initial weight of 0.78 ± 0.18 g for the standard diet and 0.80 ± 0.04 g for Herbalox®-supplemented diet were placed in to eight separate 12 L tanks. In this trial, the oil-soluble form of Herbalox® was investigated and compared with the standard diet. During the trial, water temperature, dissolved oxygen concentration, ammonia, and pH were maintained at 23.40 ± 0.54 °C, 7.16 ± 0.16 mg/l, 0.00 mg/l, 6.94 ± 0.05, respectively. This diet trial lasted for 32 days and during that period the fish were fed twice a day (9:00 and 17:00) to apparent satiation with a sinking pelleted diet. Cleaning of the tanks was carried out every three days. At the end of the trial the fish were starved for 24 h. Then, the fish from each tank were individually weighed for the measurement of final body weight. The final body weight was used for the analysis of growth performance (weight gain) including Specific Growth Rate (SGR) and Feed Conservation Ratio (FCR). For the lipid oxidation, the fish were stored in an ice box. The head and tail were removed from each fish before they were weighed. After weighing the Day 0 fish were immediately mixed with phosphate buffer (pH 7.0). The other fish were placed in separate petri dishes, which were covered with plastic lids, and stored at 4 °C for subsequent analysis at Day 2, 4, and 6.

The fish in Trial 2 were fed the antioxidant supplemented diet for 60 days. The fish in this trial had an average initial body weight of 0.54 ± 0.01 g for the standard diet, 0.48 ± 0.02 g for Herbalox®-supplemented diet and 0.48 ± 0.02 g for Vinlife®-supplemented diet. The fish were distributed into 12 square tanks and stocked at seven fish per tank. Three different diets; Standard, Herbalox® (water-soluble form)-supplemented and Vinlife®-supplemented diet were investigated. At the end of the trial, final body weight was measured individually. The mean temperature, O_2-level and pH during experimental period were 22.30 ± 0.5 °C, 7.21 ± 0.17 mg/l, and 6.94 ± 0.05, respectively. Final body weight was measured individually after 60 days of the feeding trial in order to evaluate the growth performance of the experimental fish such as SGR and FCR.

Table 1. Proximate analysis for the standard diet

Nutrient	Standard diet (%)
Protein	35.70
Fat	11.50
Moisture	3.40
Ash	11.40
Nitrogen free extract	36.40
Fiber	6.00

For the measurement of TBARS and enzyme activity, the head and tail were removed from each fish before weighing. The fish were then frozen with liquid nitrogen and then immediately ground using a mortar and pestle. In subsequent sections, the ground fish will be referred to a “fish patties”. After weighing, the Day 0 fish patty was mixed with phosphate buffer (pH 7.0). The other fish patties were put in separate petri dishes, which were covered with plastic lids, and stored at 4 °C for subsequent analysis at Day 1, 2, and 3. In Trial 2, the fish were assayed not only for lipid oxidation but also for the antioxidant enzyme glutathione peroxidase (GPx), catalase (CAT), and glutathione S-transferase (GST).

Measurement of Lipid Autoxidation. The TBARS data were expressed as malondialdehyde (MDA) equivalents (Requena *et al*. 1997). Aldehydes were the spontaneous breakdown products of lipid hydroperoxides. As an index of lipid peroxidation, the MDA concentration was measured following the method described by Buege and Aust (1978) and Lee *et al*. (1998) with some modifications. The standard curve for this method was based on a colored product resulting from the condensation of thiobarbituric acid (TBA) with malonaldehyde. When TBA was added, this causes the polar secondary product to be dissolved in the assay mixture. After heating in boiling water for appoximately 20 minutes, this material produced a pink colour. The intensity of the pink colour was directly related to the concentration of TBA-reactive substances in the original sample and this was determined by measuring the absorbance at 532 nm.

Preparation of Fish Extracts for Enzyme Assays. Preparation of fish extracts for enzyme assays was performed as described by Ahmad *et al*. (2000) with some modifications. The head and tail were removed and the fish were weighed and homogenized in chilled 0.1 M phosphate buffer (pH 7.4) containing KCl (1.17%) [w/v]. The homogenate was filtered through Miracloth^R (Calbiochem) and centrifuged at 10,000 g for 15 minutes at 4 °C. The supernatant was then taken and centrifuged again (13,000 *g*) at room temperature for 20 minutes to obtain post-mitochondrial supernatant of the fish extract, which was used for the enzyme assays.

Assay of Catalase Activity. Catalase activity was assayed by the method of Giri *et al*. (1996) and Ahmad *et al*. (2000) with some modifications. The fish extract (100 µl) was added to the reaction mixture containing 1.7 ml of 50 mM phosphate buffer (pH 7.0) and 1.2 ml of 40 mM H_2O_2 in a total volume of 3 ml. The decomposition of H_2O_2 was measured spectrophotometrically at 240 nm, using a 1.0 ml quartz cuvette in a Beckman DU 650 spectrophotometer at 25 °C. The activity was calculated using the Beer-Lambert law.

Assay of Glutathione Peroxidase Activity. Glutathione peroxidase activity was determined by a modification of the method described by Watanabe *et al.* (1996) and Athar and Ikbal (1998). The reaction mixture was consisted of 1 ml of stock solution II (100 ml of stock solution I, 0.1875 g GSH, 0.011 g NADPH), 80 µl of fish extract, 1 unit of glutathione reductase and 50 µl cumene hydroperoxide in a total volume of 1.1334 ml. Stock solution I was consisted of 50 mM potassium phosphate buffer (pH. 7.0), 3 mM EDTA, and 2 mM NaN_3. The GPx activity was assayed by following the oxidation of NADPH at 340 nm in a Beckman DU 650 spectrophotometer.

Assay of Glutathione S-Transferase Activity. Glutathione S-transferase (GST) activity was assayed according to the method of Watanabe *et al*. (1996) and Raisuddin *et al*. (1994) with some modifications. The reaction mixture was consisted of 0.5 ml of 0.2 M sodium phosphate buffer (NaPi, pH 6.5), 0.05 ml of 20 mM reduced glutathione (GSH), 0.05 ml of 20 mM 1-chloro-2,4-dinitro-benzene (CDNB), and 0.4 ml of fish extract in a total volume of 1 ml. The change in absorbance was recorded at 340 nm and the enzyme activity was calculated as nmol CDNB conjugate formed/min/g of fish tissue.

Statistical Analysis. Results are presented as the mean ± standard error mean (SEM). Differences between mean values were analyzed by one-way analysis of variance (ANOVA) followed when pertinent by a multiple comparison test (Tukey). Differences due to factors and their interactions were analyzed by two-way analysis of variance (ANOVA). The differences among specific means were evaluated through Tukey honestly significant difference (Tukey HSD) for all pairwise comparisons of means. Differences are reported as statistically significant when $p < 0.05$ (Zar 1984).

RESULTS

In the first feeding trial, SGR, WG, and FCR were not significantly affected by the different dietary treatments (Table 2). The overall survival in this experiment was high (100%) with no significant difference between dietary treatments. In the second feeding trial, fish fed the Herbalox® and Vinlife®-supplemented diets had significantly greater SGR and WG than fish fed the standard diet but FCR was not significantly different between the three diets.

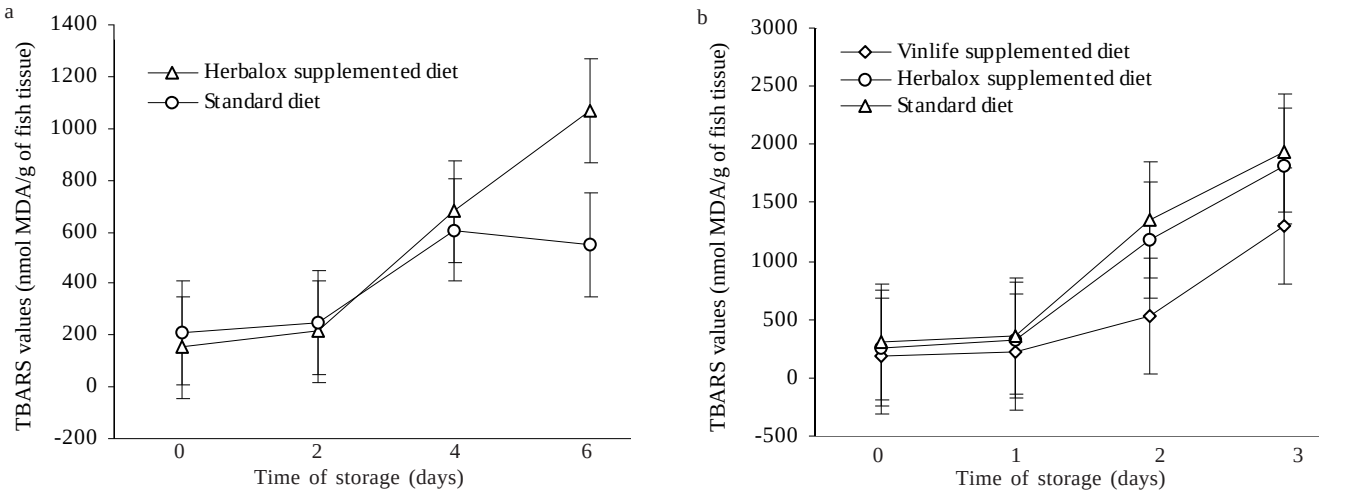

Figure 1. Formation of thiobarbituric acid reactive substances (TBARS) as an indicator of Lipid oxidation in Trial 1 (a) and Trial 2 (b). Each data point is the mean of four replicates samples. The vertical bars represent SE of the mean (SEM).

Table 2. Growth-related performance and survival rates of zebrafish after being fed the experimental diet in Trial 1 and Trial 2

Variable of measurement	Standard diet	Herbalox supplemented diet	Vinlife supplemented diet
Trial 1			
Initial weight (g)	0.78 ± 0.04	0.80 ± 0.04	
Final weight (g)	0.95 ± 0.05	0.95 ± 0.04a	
SGR	0.57 ± 0.14a	0.54 ± 0.06a	
FCR	18.18 ± 7.35a	21.62 ± 9.78a	
WG	22.57 ± 5.96a	19.37 ± 0.05a	
SR (%)	100 ± 0.00	100 ± 0.00	
Trial 2			
Initial weight (g)	0.54 ± 0.01	0.48 ± 0.02	0.54 ± 0.06a
Final weight (g)	1.05 ± 0.03a	1.06 ± 0.06a	1.04 ± 0.02a
SGR	1.09 ± 0.05a	1.31 ± 0.09b	1.30 ± 0.08b
FCR	3.70 ± 0.21a	3.68 ± 0.41a	3.45 ± 0.27a
WG	94.61 ± 0.06a	126.43 ± 11.90b	126.35 ± 9.95b
SR (%)	100 ± 0.00	100 ± 0.00	100 ± 0.00

Values are the mean ± SEM (n = 20). Means with the same superscript in the different columns were not significantly different (Tukey Test $p < 0.05$). SGR: Specific growth rate, FCR: Feed conversion ratio, WG: Weight gain, SR: Survival rate

Neither Herbalox® nor Vinlife®-supplementation could inhibit the formation of lipid oxidation products in either whole fish or fish patties (Figure 1 & Table 3). However, when the experimental diets were compared, the Herbalox®-supplemented diet showed no difference to the standard diet ($p = 0.086$) whereas the Vinlife®-supplemented diet was more successful than either the Herbalox®-supplemented diet or the standard diet in delaying lipid oxidation.

Incorporation of the novel antioxidants Vinlife® and Herbalox® into the diet of zebrafish had significant effects ($p < 0.01$) on endogenous antioxidant activities GST and CAT during post-mortem storage. However, the dietary supplements with Vinlife® and Herbalox® did not have any significant effects on GPx activity ($p = 0.275$). During the storage time, GPx and GST activity generally decreased with increasing time of storage. In contrast, CAT activity increased as the storage time increased (Figure 2). Caution must be taken when interpreting these data because there were only two replicates.

DISCUSSION

In Trial 1, SGR, WG, and FCR of the fish fed the Herbalox® (Oil-soluble form) supplemented diet were not different when compared with the standard diet. However, the most important finding is that the inclusion of the Herbalox®rosemary extract did not show any deleterious effect on growth and survival of the zebrafish. These results are in agreement with other studies conducted in several marine fish species by using Vitamin E (Gatta *et al*. 2000; Mourente *et al*. 2000; Hamre *et al*. 2004). The FCR was found to be much higher than in other studies (Gouveia & Rema 2005). There could be several explanations for this result. Firstly, the first feeding trial began when the mean initial weight of each experimental fish was 0.78 ± 0.04 g for the standard diet and 0.80 ± 0.04 g for Herbalox®-supplemented diet while in Trial 2, the mean initial weight was 0.54 ± 0.01 g for the standard diet and 0.48 ± 0.02 g for either Herbalox® and Vinlife®-supplemented diets. Secondly, most of the experimental fish used in the present study were mature from the third week of the experiment and this caused slow growth during the experimental period. The unfavorable FCR is also presumably due to the high wastage of feed so lots of feed were not consumed by the experimental fish.

In Trial 2, regardless of novel antioxidant type, antioxidant supplemented diets gave better growth to zebrafish compared to the standard diet. Diets supplemented with Herbalox® and Vinlife® gave higher SGR and WG than the standard diet but no difference was found in FCR. This result contradicts with those of Harpaz *et al*. (1998) who used another novel antioxidant, a carotenoid, in crayfish. They found that carotenoid supplementation had no effect on growth of the crayfish.

In Trial 1, it was found that dietary supplementation with Herbalox® (oil-soluble form) could not inhibit TBARS formation in the whole body of zebrafish while in Trial 2, dietary supplementation with Vinlife® more efficiently suppressed formation of TBARS in fish samples compared to

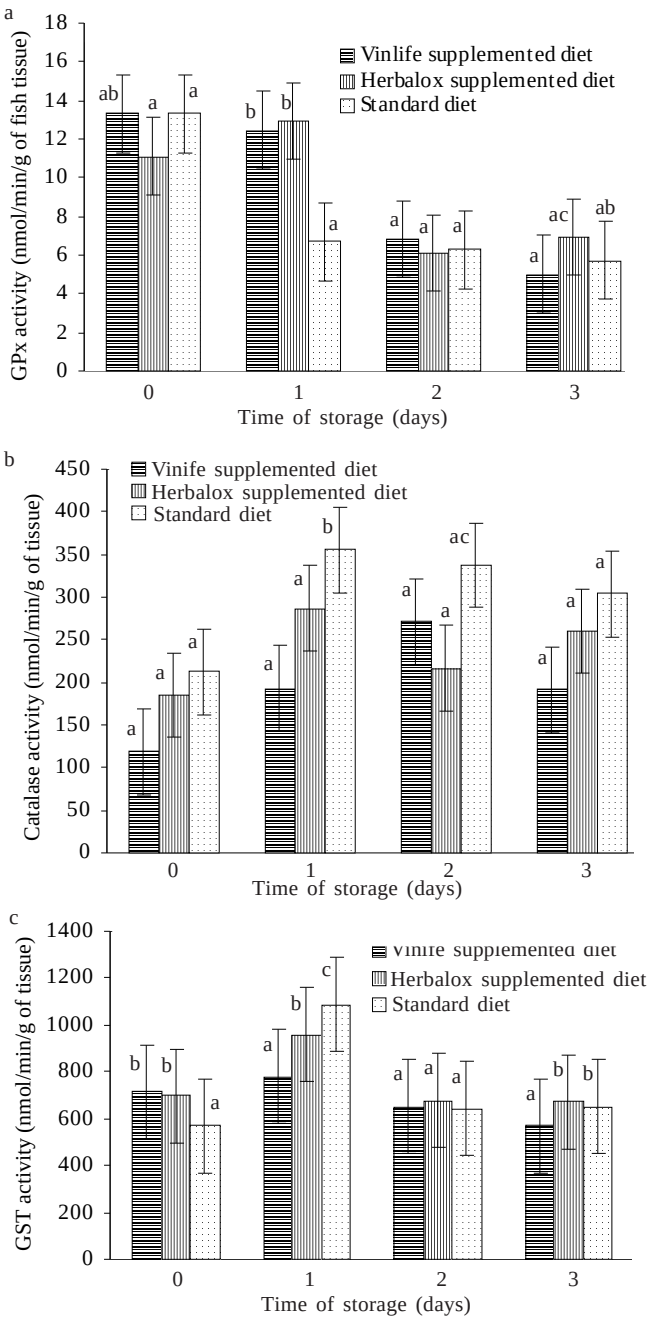

Figure 2. Effects of Dietary Antioxidants on GPx activity (a), Catalase activity (b), and GST activity (c) of Zebrafish measured at days 0, 1, 2, and 3. Each column is the mean of two replicates samples. The vertical bars represent SE of the mean. Means with the same superscripts are not significantly different (Tukey Test $p < 0.05$).

Table 3. Two-way ANOVA table of TBARS values for Trial 1 and 2 for zebrafish fed with the experimental diets

Variable of measurement	DF	p
Trial 1		
Diet	1	p = 0.086NS
Storage time	3	p < 0.01HS
Diet x time (interaction)	3	p = 0.024S
Trial 2		
Diet	2	p < 0.01HS
Storage time	3	p < 0.01HS
Diet x time (interaction)	6	p = 0.048S

Values are the mean of four replicates samples. NS: not significant ($p > 0.05$), S: significant ($p < 0.05$), HS: highly significant ($p < 0.01$)

the Herbalox® and the standard diet. This result is in agreement with that of Rababah *et al*. (2004) who found that addition of a novel antioxidant such as a grape seed extract could effectively reduced the accumulation of TBARS values.

Vinlife® grape seed extract is high in polyphenolic antioxidants and this may explain its effectiveness in retarding lipid oxidation in the fish patties. Grape seed extracts containing a large amount of polyphenolic and phenolic compounds may inhibit the formation of free radicals during the initiation step or interrupt the propagation step by acting as electron donors or by scavenging the free radicals (Kaur & Kapoor 2004). As far as the phenolic content of grape seed extract and rosemary extract is concerned, our results contradict with those of Rababah *et al.* (2004) who found that rosemary had a relatively higher amount of phenolic compounds compared to grape seed extract. Based on the results obtained, the stronger antioxidant activity of Vinlife® suggests that Vinlife® has greater antioxidant potential than Herbalox®. This difference in antioxidant potential may or may not be due to differences in phenolic content. The polyphenols found in Vinlife® are mainly proanthocyanidins (monomeric flavan-3-ols, specially (+) – catechin, (-) – epicatechin, (+/-) gallocatechin, (-) – epicatechin 3-0-gallate; and their oligomers and polymers (http://www.tarac.com.au), while the chemical constituents of Herbalox® are rosmarinic acid (RA), caffeic acid (CA), chlorogenic acid, carnosolic acid, rosmanol, carnosol, various diterpenes, rosmari-diphenol, rosmariquinone, carnosic acid, and various esters (Hudson 1990; Madhavi *et al.* 1995; Al-sereiti *et al.* 1999).

In the second trial, formation of TBARS at day 0 was higher compared to the first trial. This finding indicates that ROS-initiated lipid oxidation had occurred prior to the storage of the sample, apparently during the preparation of sample. Induced lipid oxidation already occurred when the sample was ground using a mortar and pestle in which the oxygen came into contact with the exposed sample surface. In addition, grinding disrupted the membranes and facilitated the release of iron from myoglobin. This, in turn could have induced lipid oxidation in the sample prior to the storage.

The TBARS values of all dietary treatments after three day's storage were found to be significantly greater than the TBARS values at day 0. The TBARS values of all treatments increased 7-fold with the highest values found in the fish fed the standard diet. More than 50% of the fish tissue contents was composed of PUFAs, which makes fish tissue more susceptible to oxidative attack. Kolaily and Keha (1999) stated that higher levels of TBARS were present in freshwater fish compared to seawater fish. This is might be due to the seawater fish are more resistant to oxidative stress.

The overall antioxidant activities of GPx, CAT, and GST were higher compared to the other published reports. Kolaily and Keha (1999) reported that CAT and GPx activities in the liver of freshwater trout were 3.54 ± 0.55 nmol/min/g of tissue and 22.47 ± 2.55 nmol/min/g of tissue, respectively. They also investigated GST activity in the liver of freshwater trout and found the activity was 18.49 ± 2.25 nmol/min/g of tissue.

The novel dietary antioxidants Vinlife® and Herbalox® had significant effects on CAT activity during three days of storage. The CAT activity of the fish fed the Vinlife®grape seed extract was lower compared to the fish fed the Herbalox® and the standard diet. In this respect, it is interesting to note that the low CAT activity was probably due to the lower level of oxidative stress, which was determined by the TBARS value of the fish sample.

Decreased GPx activities were observed in the fish fed the Vinlife®-supplemented diet and the standard diet during subsequent storage time. This was probably due to deterioration of the GPx enzyme during post-mortem storage. Increased oxidative attack during storage can overcome GPx activity leading to its deterioration. The GPx deterioration was in agreement with the data published by Watanabe *et al.* (1996) who investigated GPx activity in skipjack tuna muscle during post-mortem storage. They found that GPx activity deteriorated in skipjack tuna muscle over five day's storage. The longer the post-mortem storage the lower the GPx activity found tuna muscle.

The existance of PUFAs in fish sample can undergo spontaneous free radical-initiated lipid oxidation resulting in the formation of hydrogen peroxides. Both CAT and GPx can neutralize the damaging effect of hydrogen peroxides. A low level of GPx activity can be compensated for by an increased CAT activity. When enhanced CAT activity is sufficient to inhibit the formation of lipid oxidation products, the low GPx activity is favored for fish tissue. Another reason is that when the total amount of PUFA target is decreased, fish tissues favor the least energy expensive enzyme. Catalase requires no cofactors for its activity while GPx requires glutathione, which is oxidized upon reaction with GPx and must be recycled by a NADPH-consuming glutathione reductase (Janssens *et al*. 2000; Chang *et al.* 2005). The results of the present study suggest that CAT is more important in zebrafish than GPx.

The effect of dietary Vinlife® supplement on GST activity was statistically significant compared to the standard diet ($p < 0.05$). There could be several reasons for this. The most likely is that decreased GST activity is due to proteolysis of the GST enzyme post mortem. This finding may suggest that the storage time affects the effectiveness of the dietary supplement Vinlife® to suppress any overt signs of toxicity in zebrafish. More studies are required to solve the contradictive results found in this present experiment. Effects of different dietary treatment and storage time on lipid oxidation and antioxidant enzyme activities should be examined in order to find more reliable results.

Comparing the two novel dietary antioxidants, Vinlife® shows stronger antioxidant properties compared to Herbalox®. Data obtained from this present study indicated the protective effect of dietary Vinlife® supplement against lipid oxidation. This novel antioxidant showed significant effects on antioxidant enzyme activities including CAT and GST, in which the activities of the enzymatic antioxidants CAT and GST were sufficient to inhibit and to maintain the low rate of lipid oxidation during subsequent storage time at 4 °C. A low level of GPx activity can be compensated for by an increased CAT activity. The low GPx activity is favored for fish tissue because CAT activity is sufficient to inhibit the formation of lipid oxidation products. Dietary supplementation with Vinlife® did

not have any deleterious effects on growth performance of zebrafish. Therefore, Vinlife® supplementation could be useful for keeping the oxidative stability of the valuable n-3 PUFAs content in fish.

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